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13. ABSTRACT (Maximum 200 Words)

In our laboratory we investigate genes that are critical for mammary gland development with the ultimate goal of understanding how these key pathways may be disrupted during breast cancer progression. Previously we reported that p190B RhoGAP is essential for ductal morphogenesis. The studies outlined in this proposal are aimed at further elucidating the role of p190B and Rho signaling in all stages of mammary gland development and the molecular mechanisms through which p190B acts to influence development of the mammary gland. For this purpose, four lines of tetracycline-regulatable p190B overexpressing mice have been generated. P190B overexpression during virgin mammary gland development resulted in abnormal TEB structures, increased branching, and thickened stroma surrounding the ducts. In addition, an MCF7 breast cancer cell culture model has been developed in which siRNA oligos are used to specifically downregulate p190B expression. This system will be used to delineate the molecular mechanisms through which p190B influences cell proliferation, survival, and migration. Ultimately, the role of p190B in breast cancer progression will be investigated using p190B overexpressing and deficient mice crossed to a well-established mouse model of breast cancer, the MMTV-ErbB2 line of mice.

14. SUBJECT TERMS
p190B RhoGAP, terminal end buds, ductal morphogenesis, mammary gland development, tumor biology, cell signaling

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Introduction

In our laboratory we investigate genes and signaling pathways that are critical for mammary gland development and function with the ultimate goal of understanding how these key pathways may be disrupted during breast cancer progression. P190B RhoGAP was identified as a gene that is preferentially expressed in the terminal end buds of the developing mammary gland. Previously we reported that loss of p190B function completely prevents ductal outgrowth in the mammary gland. This result suggests that regulated signaling of the Rho pathway downstream of p190B is essential for proper development of the mammary gland. The studies outlined in this proposal are aimed at further elucidating the role of p190B and the Rho signaling pathway in all stages of mammary gland development and the molecular mechanisms through which p190B acts to influence the different developmental stages in the mammary gland. The role of p190B and the Rho signaling pathway in breast cancer progression will also be investigated using a well-established mouse model of breast cancer, the MMTV-ErbB2 line of mice.

Body

Task 1. To elucidate the role of p190B in mammary gland (MG) development in p190B deficient and tetracycline (Tet)-regulatable p190B overexpressing mice (Months 1-24).

When this fellowship application was prepared in May of 2002, two founder tetracycline (Tet)-regulatable p190B mice had been generated in Dr. Rosen's laboratory. However, further characterization revealed that these founder mice failed to transmit the transgene to their offspring. In addition, the construct used to generate these mice resulted in an unusually low transgenesis rate. It is possible, despite the presence of Tet-regulatable sequences in the construct, that a low level of p190B was expressed in transgenic embryos due to an essential minimal cytomegalovirus (CMV) promoter present in the Tet construct. Because this transgenic construct is targeted to every tissue, leaky expression of p190B during embryogenesis could result in early embryonic death. This would account for the low transgenesis rate that was observed. Therefore, it was necessary to generate new Tet-regulatable p190B founder mice. For this reason, the experiments proposed in Task 1 have been slightly delayed while a new transgenic construct and founder mice were generated. It is still expected, however, that the experiments will be completed within the 24-month time frame originally proposed for Task 1.

To generate Tet-regulatable p190B transgenic mice a new construct containing the p190B cDNA linked to luciferase by an inter-ribosomal entry site (IRES) was designed (Figure 1). The Tet-regulatable IRES-luciferase construct was kindly provided by Dr. Lewis Chodosh. Addition of the IRES-luciferase allows rapid and quantitative screening of transgene expression. Expression of 190B and luciferase from the newly generated construct was confirmed in cell culture using MCF7 Tet-On cells (generously provided by Dr. Adrian Lee). Injection of this construct into embryos yielded seven founder mice that were confirmed to be positive for the transgene by Southern blot analysis (Figure 2).

Founder mice were bred to MMTV-rtTA mice to generate bigenic mice for analysis of transgene expression. Five week-old female bigenic mice from these matings were treated with the tetracycline analog doxycycline (Dox) at 2 mg/ml in their drinking

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water for three days to induce transgene expression. Dox-treated single MMTV-rtTA and Tet-regulatable p190B transgenic mice as well as untreated bigenic female mice served as controls. Following treatment, mammary glands were dissected from the mice and analyzed for luciferase activity, p190B transgene expression by RT-PCR, and morphological changes by whole-mount mammary gland analysis. Four of the founder lines of mice, lines 6670, 6671, 6674, and 6667 transmitted the transgene to their pups and expressed the p190B transgene as determined by RT-PCR and luciferase assays. Interestingly, after only three days of p190B transgene induction, a striking effect on the morphology of the terminal end buds (TEBs) in the developing glands was observed. Whole-mount analysis of mammary glands from Dox-induced bigenic females revealed aberrant branching off the neck region of the TEBs and abnormal TEB morphology (Figure 3). Histological analysis of hematoxylin and eosin stained tissue sections from these glands further demonstrated the extent of disruption of the TEB structure (Figure 4). Immunohistochemical staining for the cell proliferation and death markers, Ki67 and active caspase-3, respectively, showed that the aberrant branches off the TEBs were highly proliferative and few cells were undergoing apoptosis (Figure 5).

As proposed in Task 1e, I have begun to investigate the effects of p190B overexpression throughout virgin mammary gland development. For this study, 4 bigenic and 4 age-matched wildtype littermates from line 6671 were treated with doxycycline from 5.5-9.5 weeks of age. At the end of the treatment period, the glands were collected and transgene expression was examined by performing a luciferase assay and RT-PCR (Figure 6 and data not shown). Both assays confirmed expression of p190B in all four bigenic mice. Analysis of whole-mounted and H&E stained mammary glands revealed an increase in ductal branching, abnormal TEB structures, and thickened stroma surrounding the ducts and TEBs (Figures 6 and 7). These morphological changes are consistent with the increased budding off the necks of the TEBs that was observed in this line after 3 days of transgene induction. Currently, I am in the process of obtaining age-matched non-induced bigenic littermate controls for this study. Several litters must often be screened before sufficient numbers of bigenic female mice are obtained since the expected frequency of these mice is only 1/8. In addition, this experiment is being repeated in a second inducible line 6667. As proposed in Task 1e, I am also investigating whether p190B overexpression affects pregnancy, lactation, and involution. Once these studies are completed, the tissues will be used to investigate the mechanisms of p190B action in virgin mammary gland development, pregnancy, lactation, and involution as proposed in Tasks 1d and 1f.

The results described above are consistent with the loss of function studies performed in the p190B+/- mice previously reported by our laboratory (Chakravarty, G., et al., 2003 *Molecular Endocrinology* 17(6): 1054-1065). While loss of p190B function completely prevented development of the mammary gland, overexpression of p190B during mammary gland development may increase branching of the ducts by enhancing proliferation and cell survival. These changes as well as the altered morphology of the TEBs may also affect migration of the mammary epithelial cells into the fat pad during virgin development. The experiments proposed in Tasks 1d and 1f will help to elucidate the molecular mechanisms through which p190B influences mammary gland development and function.

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In Tasks 1b and 1c, I proposed to treat p190B+/- and Tet-regulatable p190B transgenic mice with an intravenous injection of IGF-1 (Insulin-like growth factor-1) to determine whether the effects of p190B expression on mammary gland development are due to p190B dependent modifications of IGF1-R (Insulin-like growth factor-1 receptor) signaling. Our laboratory has now published that expression of IRS (Insulin receptor substrate) proteins, the immediate downstream effectors of IGF1-R, is significantly reduced in TEBs of the p190B+/- mice (Chakravarty, G., et al., 2003 *Molecular Endocrinology* 17(6): 1054-1065). The reduced IRS expression levels correlated with reduced proliferation in the TEBs. These experiments were performed in untreated mice, which demonstrates that it is not necessary to inject the mice with IGF-1 in order to observe alterations in the IGF1-R signaling pathway in p190B deficient mice. In addition, it has proven to be difficult to detect changes in IGF1-R signaling in situ in the mammary glands of mice injected with IGF-1. This is likely to be due to the fact that physiological levels of circulating IGF-1 are high in mice and fasting prior to IGF-1 injection may not be sufficient to reduce these levels. For these reasons I have decided to perform Tasks 1c and 1d in the absence of intravenous injections of IGF-1.

Task 2. *To investigate the role of p190B in breast cancer progression using both p190B loss and gain of function mouse models (Months 12-36).*

In Task 2a, I proposed to mate p190B+/- mice to MMTV-polyoma middle T (PyMT) mice to analyze the effects of decreased p190B expression on tumor latency, growth, and metastasis. I have now decided to investigate the affects of p190B deficiency on tumor progression by mating the p190B+/- to the MMTV-ErbB2 mice as I proposed to do with the Tet-regulatable p190B transgenic mice in Task 2d. The rationale behind this change is due to significant concern that loss of only one allele of p190B will not be sufficient to impact tumorigenesis in the presence of such a strong tumorigenic stimulus as the polyoma middle T. Because these studies require a substantial number of animals and time, I believe a better experiment is to use the MMTV-ErbB2 model. ErbB2 is not as strong of an oncogenic stimulus as the PyMT since the MMTV-ErbB2 mice demonstrate a longer tumor latency and slightly lower tumor incidence when compared to the MMTV-PyMT mice. Currently, I am in the process of obtaining the MMTV-ErbB2 mice for these studies. Once I have finished characterizing the Tet-regulatable p190B transgenic mice I will begin the experiments proposed in Tasks 2d, 2e, and 2f.

Task 3. *To delineate the molecular mechanism by which p190B facilitates tumor cell migration and invasion using a Tet-regulatable p190B overexpressing MCF-7 breast cancer cell line (Months 1-12).*

For the experiments described in Task 3, I proposed to generate Tet-regulatable p190B overexpressing MCF7 breast cancer cells. To do this I took advantage of the Tet-regulatable p190B-IRES-luciferase construct that was used to create the transgenic mice in Task 1. This construct was shown to be functional in MCF7 Tet-On cells (kindly provided by Dr. Adrian Lee) by transient transfection and analysis of HA-tagged p190B by Western blotting and luciferase assays. To generate stably transfected MCF7 Tet-On cells the p190B-IRES-luciferase construct was co-transfected with a plasmid containing a puromycin resistance cassette. Puromycin resistant clones were isolated and expanded.

To determine whether the clones expressed p190B and luciferase, Dox (2 μg/ml) was added to the growth medium for 48 hours. Untreated cells served as controls for inducibility of the transgene. Cell extracts were prepared, and initially, luciferase assays were performed. 13/52 clones showed luciferase activity. However, when Western analysis for HA-tagged p190B was performed 0/13 of the clones showing luciferase activity were found to express HA-tagged p190B (Figure 8). This was surprising since this construct showed expression in both transient transfection experiments and in vivo. Further analysis the MCF7 Tet-On cells revealed that they express readily detectable levels of endogenous p190B as well as equivalent levels of the related p190A RhoGAP. It is likely that expression of these proteins, which are critical regulators of numerous cellular processes including cell shape, movement, proliferation, and survival, is tightly regulated. Thus, the cells may not tolerate overexpression of p190B, and upon induction the HA-tagged p190B may be rapidly degraded.

Since several attempts to generate MCF7 cells stably overexpressing p190B have been unsuccessful, I have decided to take an alternative approach in which siRNA oligos are used to specifically downregulate endogenous p190A or p190B in the MCF7 cells. It is unclear whether p190A and p190B will have overlapping or unique functions in these cells, and therefore, it is important to perform the experiments in cells in which either p190A or p190B expression is decreased. I have already obtained and tested several siRNA oligos to downregulate p190A and p190B, and I have identified three oligos that work efficiently for each protein (Figure 9). Thus, the experiments outlined in Task 3 will now be performed using p190A and p190B deficient MCF7 cells instead of p190B overexpressing cells. Due to the unforeseen difficulties in obtaining Tet-regulatable p190B overexpressing cells and establishment of the siRNA system in the MCF7 cells, the experiments proposed in Task 3 have been delayed. I am now in the process of beginning migration assays and microscopy studies with siRNA treated cells as outlined in Tasks 3a and 3b.

While I was working to establish cells in which p190B expression can be manipulated, I began to examine the effects of IGF-1 and estrogen stimulation on cell migration and signaling downstream of p190B (and now p190A) as proposed in Task 3d. For these experiments, MCF7 cells were cultured in serum and phenol red-free medium overnight prior to stimulation with IGF-1 (80 ng/ml), estrogen (1nM) or both factors. In the MCF-7 cells in response to a short time course of IGF-1 and estrogen stimulation, endogenous p190B and p190A are rapidly and transiently inactivated (Figure 10). IGF-1 treatment alone results in an attenuated activation of p190B and p190A (Figure 11 and data not shown). In contrast, stimulation with only estrogen results in a pattern of activation and de-activation of p190B and p190A that is distinct from that induced by IGF-1 (Figure 11). A downstream target of Rho kinase and key player in actin cytoskeletal reorganization, cofilin, is regulated in response to both IGF-1 and estrogen treatment. The unique phosphorylation patterns of p190A and p190B observed in response to the different stimuli suggest that IGF-1 and estrogen may be capable of cooperating or acting independently to affect cellular processes downstream of the RhoGAPs. The next step is to perform migration assays and microscopy to examine the cytoskeletal changes in response to IGF-1 and estrogen as proposed in Tasks 3a and 3b. These experiments will also be repeated in siRNA treated cells.

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An interesting an unexpected finding was that p190A showed the same phosphorylation patterns as p190B in response to the different treatments. This result suggests that at least in the MCF7 cells p190A and p190B may have overlapping functions with respect to the effects of IGF-1 and estrogen on signaling pathways downstream of the RhoGAPs. Several reports in the literature have demonstrated unique functions for p190A and p190B in a variety of developmental processes. Thus, the overlap in activities of the two family members in the MCF7 cells was not predicted. Furthermore, loss of p190B in vivo completely blocks mammary gland development, which indicates that p190A cannot functionally compensate for p190B in the developing mammary gland. I have now screened a panel of ten breast cancer cell lines for expression of p190B and p190A, and all were found to express both proteins. This finding raises the important question of whether both family members can contribute to breast cancer progression while perhaps only p190B may be critical for mammary gland development. Currently, experiments are ongoing in our lab to determine whether p190A also plays an important role in mammary gland development in vivo.

Key Research Accomplishments

- Four lines of Tet-regulatable p190B overexpressing mice have been generated and characterized.
- Striking morphological abnormalities have been detected in the developing mammary glands of Tet-regulatable p190B overexpressing lines after short term (3 days) and long term (4 weeks) of p190B induction.
- Both p190A and p190B may be important for breast cancer progression while p190B alone may be critical for normal mammary gland development.
- IGF-1 and/or estrogen stimulation of MCF7 breast cancer cells results in unique phosphorylation changes in p190B and p190A.
- Three specific siRNA oligos have been identified for both p190B and p190A. These oligos will be used to knockdown expression of p190B and p190A in MCF7 cells to study the role of each protein in cell migration, proliferation, and survival.

Reportable Outcomes
None

Conclusions
I have now established four lines of Tet-regulatable p190B overexpressing transgenic mice. Strikingly, these mice show abnormal TEB structures after only three days of transgene induction and an even more pronounced phenotype when p190B is overexpressed throughout virgin mammary gland development. Generation of these mice was a critical first step toward my goal of further elucidating the role of p190B in all stages of mammary gland development as well as determining the molecular mechanisms through which p190B acts to regulate mammary gland development and function. Importantly, these mice will be used to investigate the role of p190B and the Rho signaling pathway in breast cancer progression as little is known about the contribution of Rho signaling to breast tumorigenesis. I have also made significant progress in

developing an in vitro system using siRNA to specifically downregulate either p190A or p190B expression in the MCF7 breast cancer cell line. MCF7 cells are one of very few cell culture models that are responsive to both IGF-1 and estrogen treatment. Establishment of this model system will allow me to dissect the effects of IGF-1 and estrogen stimulation on p190B and downstream signaling molecules.

References:
Chakravarty, G., et al., "P190-B RhoGAP Regulates Mammary Ductal Morphogenesis" (2003) *Molecular Endocrinology* 17(6): 1054-1065.

Appendix: Current contact information
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Figure 1: Generation of Tet-regulatable p190B overexpressing mice. A. p190B IRES-luciferase construct. B. Construct was tested by transient transfection followed by Dox induction (2μg/ml) in MCF7 Tet-On cells. Lysates were analyzed for luciferase activity. C. Western blot analysis was performed to detect the HA-tagged p190B protein in the lysates from the transiently tranfected MCF7 Tet-On cells.
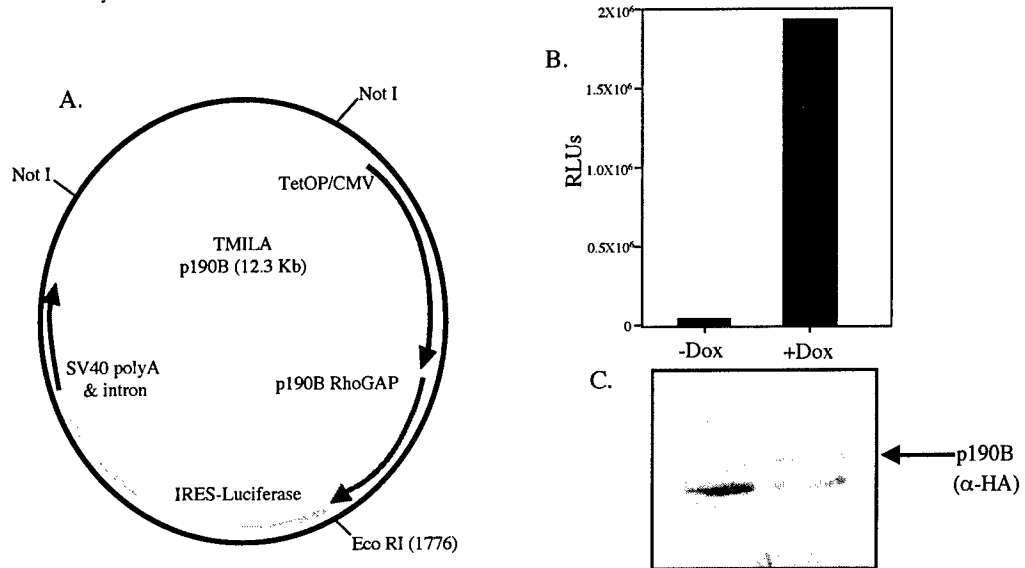
Figure 2: Southern blot analysis to confirm founder mice generated by injection of p190B IRES-luciferase construct into embryos. 7/24 founder mice were positive for the transgene. Only positive founders are shown.
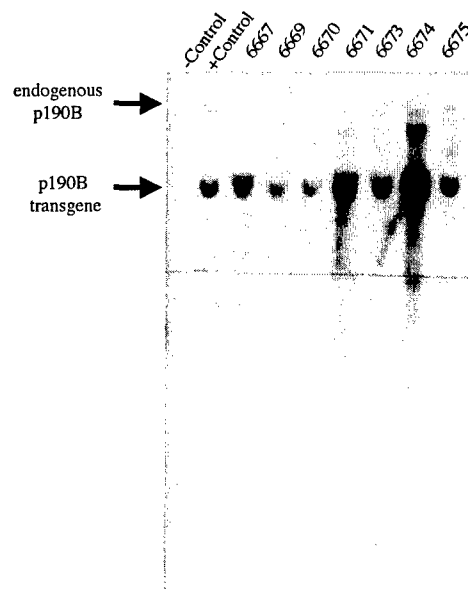
Figure 3: A.Wholemount analysis of mammary glands from bigenic females +/- Dox (2mg/ml) for 3 days. Arrows indicate TEBs. Notice the budding off the necks of the TEBs in the bigenic +Dox, which is not seen in the bigenic -Dox. B. RT-PCR was performed to confirm expression of the p190B transgene after 3 and 7 days +Dox. MTB mice served as a negative control and L19 RT-PCR was performed to confirm that the cDNA could be transcribed. -RT controls were negative (data not shown).
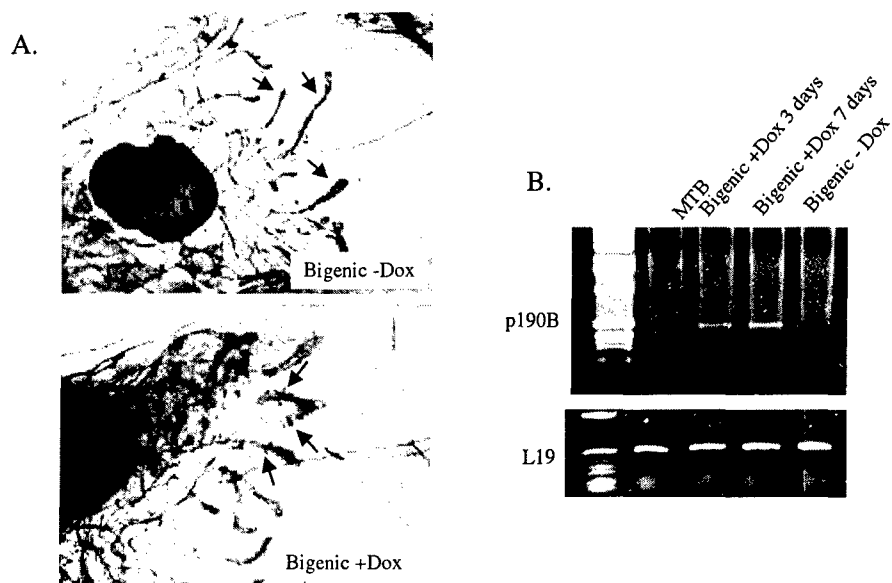
Figure 4: H&E and Masson's trichrome staining of TEBs from +Dox (panels B and D) and -Dox (panels A and C) treated Bigenic mice. Abnormal TEB structures with aberrant budding (double headed arrow) were observed in the +Dox treated mammary glands. Arrows indicate the abnormal presence of stroma at the tip of the TEB in the +Dox treated mammary glands. Masson's trichrome staining was performed to mark the stroma around the TEBs.
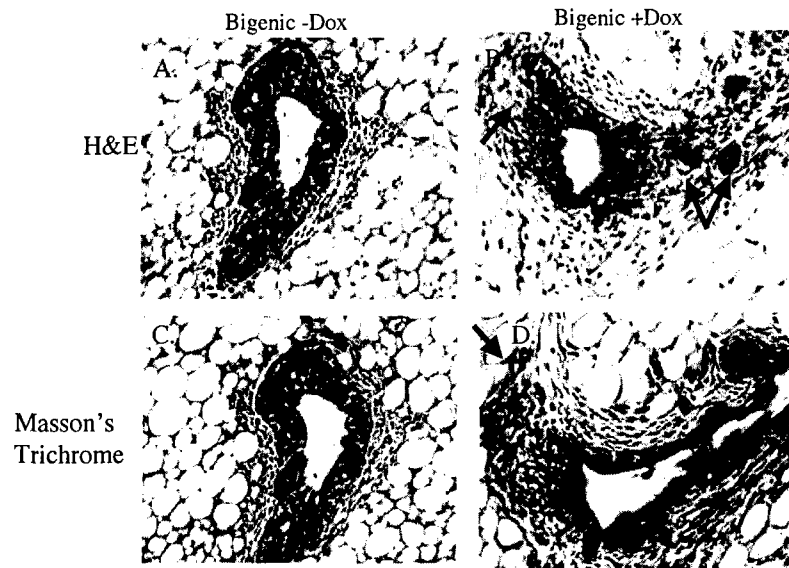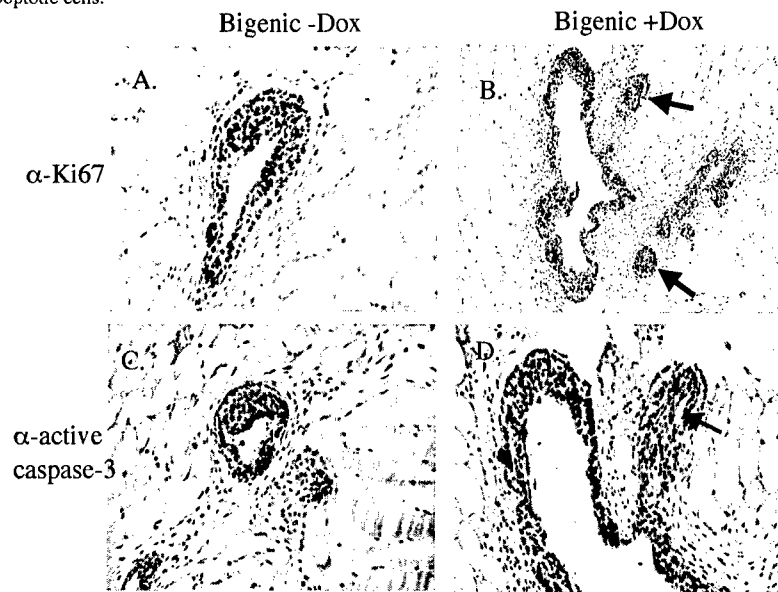
Figure 5: Ki67 (panels A and B) and anti-active caspase-3 (panels C and D) immunohistochemical staining was performed on + Dox treated (panels B and D) and -Dox treated (A and C) mammary glands to detect proliferating and apoptotic cells, respectively. Arrows indicate aberrant buds off the TEB which contain many proliferative, but few apoptotic cells.

Bigenic -Dox   Bigenic +Dox

α-Ki67

α-active
caspase-3

Figure 6: Wholemount analysis of mammary glands from a wildtype age-matched littermate (panel A) and bigenic female after 4 weeks of Dox treatment. Arrows indicate abnormal branching and TEBs in the bigenic mammary gland. C. RT-PCR was performed to confirm p190B transgene expression in the mammary glands of bigenic females after Dox treatment. Age-matched wildtype littermates were negative for p190B transgene expression in the mammary gland. L19 was performed as a postive control for the RT reaction.
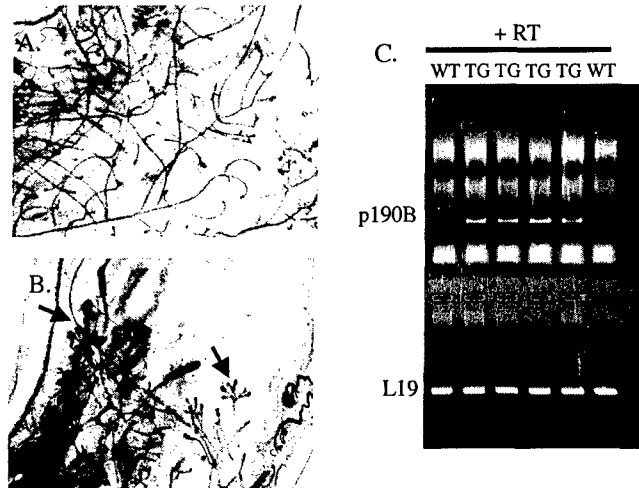
Figure 7: H&E staining of mammary glands from Bigenic (panels B and D) and wildtype control littermates (panels A and C) treated with Dox for 4 weeks. Panels A and B show ductal structures and panels C and D show TEBs. Arrows indcate abnormal stroma surrounding the duct and morphologically abnormal TEB in the Bigenic mammary gland.
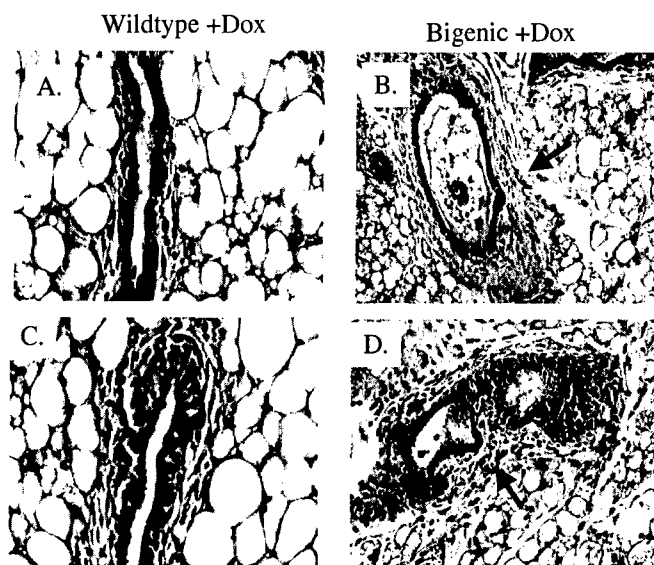
Wildtype +Dox   Bigenic +Dox

Figure 8: Stable overexpression of p190B in MCF-7 may not be possible. A. Construct used in attempt to generate Tet-regulatable p190B overexpressing MCF7 cells. B. Western analysis for endogenous p190B and HA-tagged p190B performed on lysates prepared from puromycin-resistant MCF7 clones (obtained after transfection of the plasmid shown in panel A) +/- Dox. 5 representative clones are shown. All clones express endogenous p190B, whereas none of the clones expressed the HA-tagged p190B. C. Luciferase assays were performed on the 5 representative clones from panel B all of which show activity. In total, 13/52 clones were positive for luciferase, but none expressed the HA-tagged p190B.
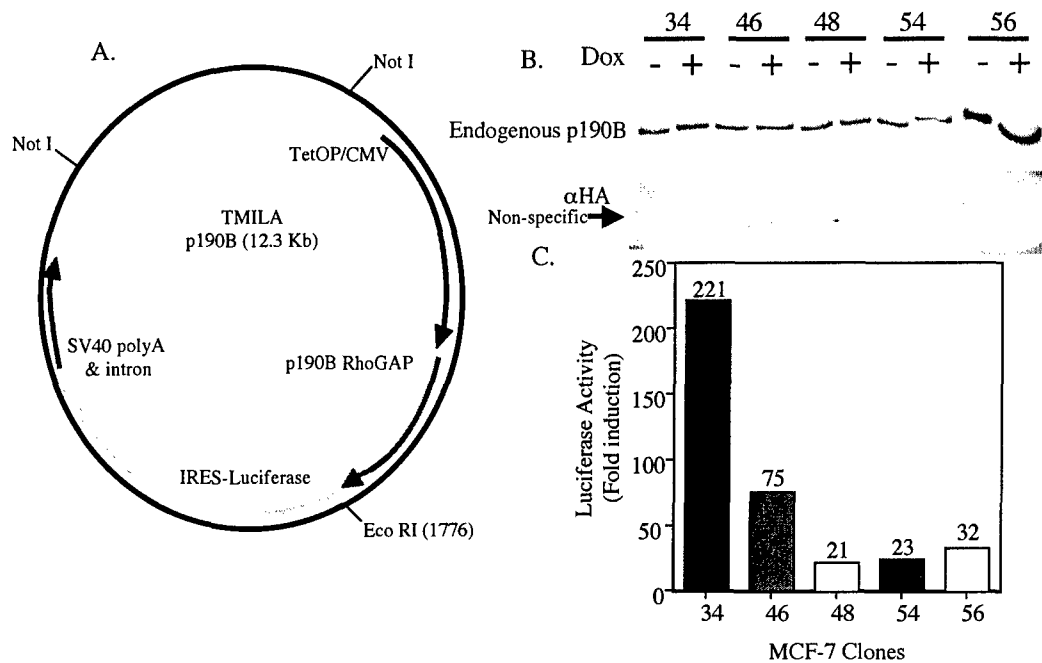
Figure 9: siRNA oligos that specifically knockdown p190B or p190A in MCF7 cells. MCF7 cells were transfected with 1 of 4 unique oligos (100nM) designed to knockdown either p190A or p190B. After 72 hours lysates were prepared and analyzed by Western blotting for p190B or p190A. No oligo or a non-specific control (CTL) oligo were used as negative controls. Panels A and B show Westerns of cell lysates from MCF7 cells transfected with siRNA oligos against p190A and probed with an antibody to p190A or p190B (panels A and B, respectively). Panels C and D are Westerns performed with lysates from MCF7 cells transfected with siRNA oligos against p190B and probed with an antibody to p190B or p190A (panels C and D, respectively). Results indicate 4 specific oligos for p190A and 3 specific oligos for p190B
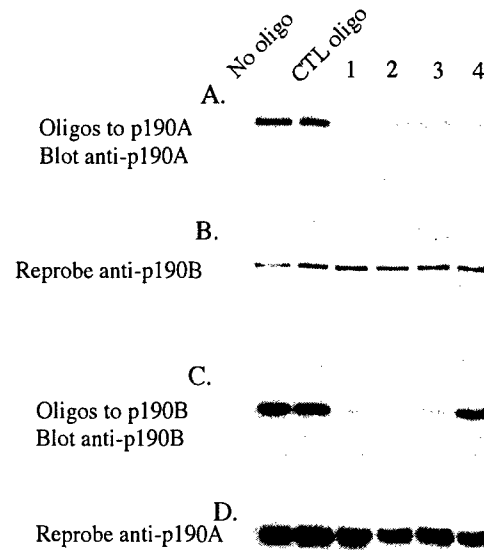
Figure 10: IGF-1 and estrogen (E₂) cooperate to regulate p190B and p190A signaling. MCF7 cells were starved overnight in serum free and phenol red free medium. E₂ and IGF-1 were added and lysates were collected at the times indicated. Panels A and B are Western blots for p190B or p190A. In panels C and D, an antibody against phospho-tyrosine (pTyr) was used to immunoprecipitate tyrosine phosphorylated proteins. These samples were then blotted for either p190B or p190A. Note that the phosphorylation patterns of p190A and p190B resulting from IGF-1 and E₂ treatment are similar. Panel E is a Western blot for phospho-cofilin, which is a downstream target of p190B.
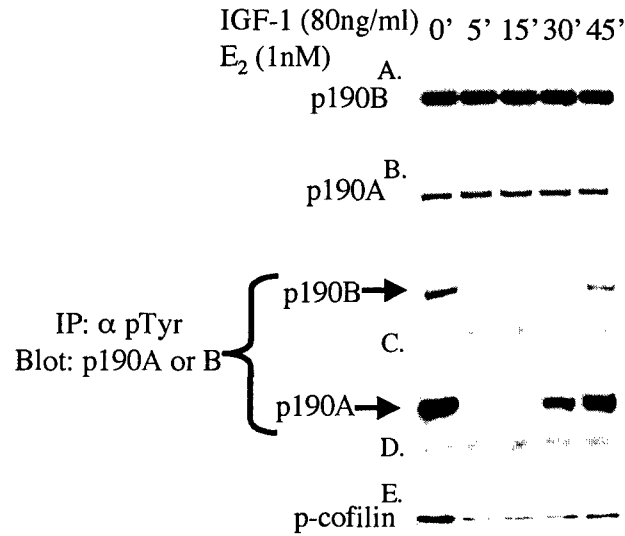
Figure 11: IGF-1 or estrogen treatment alone results in a unique phosphorylation pattern for p190B and p190A. MCF7 cells were starved overnight in serum free and phenol red free medium. IGF-1 (panel A) or E_2 (panel B) was added to the cells and lysates were collected at the times indicated. Immunoprecipitation was performed using an anti-pTyr antibody. Western blotting for either p190B or p190A was performed using these samples. Note that the phosphorylation pattern of p190A and p190B is distinct from that observed when cells were treated with both IGF-1 and E_2.

A.   IGF 80ng/ml   0' 5' 15' 30' 45'   B.   1nM E_2 0'   5' 15' 30' 45'

IP: pTyr
Blot: p190B

p190B

p-Cofilin

IP:p-Tyr
Blot:p190B

IP:p-Tyr
Blot:p190A